



Apple and blackcurrant polyphenol-rich drinks decrease postprandial glucose, insulin and incretin response to a high-carbohydrate meal in healthy men and women^{☆,☆☆}

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Abstract

Postprandial glycemic responses to meals are inhibited by polyphenol-rich plant foods. Combinations of polyphenols may be particularly effective through complementary mechanisms. A randomized, controlled, double-blinded cross-over trial was conducted in healthy volunteers to test the hypothesis that apple and blackcurrant polyphenol-rich drinks would reduce postprandial blood glucose concentrations. Secondary outcomes included insulin and glucose-dependent insulinotropic polypeptide (GIP) secretion. Twenty men (mean age 26 y, SD 8) and 5 postmenopausal women (mean age 57 y, SD 3) consumed a placebo drink (CON) and 2 polyphenol-rich drinks containing fruit extracts: either 1200 mg apple polyphenols (AE), or 600 mg apple polyphenols+600 mg blackcurrant anthocyanins (AE+BE), in random order with a starch and sucrose meal. Incremental areas under the curve (iAUC) for plasma glucose concentrations were lower following AE+BE over 0–30 and 0–120 min compared with CON; mean differences (95% CI) –32 mmol/L·min (–41, –22, $P<.0005$) and –52 mmol/L·min (–94, –9, $P<.05$), respectively. AE significantly reduced iAUC 0–30 min (mean difference –26 mmol/L·min, –35, –18, $P<.0005$) compared with CON, but the difference over 120 min was not significant. Postprandial insulin, C-peptide and GIP concentrations were significantly reduced relative to CON. A dose response inhibition of glucose transport was demonstrated in Caco-2 cells, including total and GLUT-mediated transport, and SGLT1-mediated glucose transport was strongly inhibited at all doses in *Xenopus* oocytes, following 10 min incubation with 0.125–4 mg apple polyphenols/ml. In conclusion, ingestion of apple and blackcurrant polyphenols decreased postprandial glycemia, which may be partly related to inhibition of intestinal glucose transport.

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1. Introduction

According to the World Health Organization, 9% of the world's adult population suffers from diabetes, and the rising prevalence of type 2 diabetes (T2D) is predicted to reach exceptional levels [1]. Regular exposure to diets with a high glycemic load (a measure of the overall blood glucose-raising effect of a serving of a food) may contribute to postprandial hyperglycemia, a risk factor for T2D and

endothelial dysfunction [2–5]. Strategies to control chronic postprandial hyperglycemia by optimizing the functionality of foods would strengthen efforts to reduce the risk of developing T2D in the general population. The physico-chemical composition of certain fruits, including cell wall structure [6] and polyphenolic constituents [7,8], may help to delay starch and disaccharide digestion and glucose absorption following a carbohydrate-containing meal or beverage. In vitro studies suggest that some berry anthocyanins and apple polyphenols are effective inhibitors of digestive enzymes, α -amylases and α -glucosidases [9–13]. Furthermore, polyphenols found in berries and apples inhibit the action of intestinal glucose transporters SGLT1 and GLUT2 [8,14–18]. Human data is limited; however, randomized controlled trials (RCTs) have shown that berries [19–22] and apple products [8,23,24] reduced postprandial glucose concentrations following consumption of either starch, glucose or sucrose loads. However, the majority of study designs have administered the whole fruit or fruit product, e.g. juice, making it difficult to identify the effects of the polyphenols relative to other aspects of the fruit composition. Confounding factors, including the degree of mastication, variability in the food matrix, and interactions with macronutrients, could influence the release of polyphenols and digestible carbohydrate from plant cell walls in whole fruit test meals

Abbreviations: AE, Apple extract; AE+BE, Apple and blackcurrant extracts; iAUC, Incremental area under the curve; C_{max} , Maximum concentration; CON, Control treatment; DVP-SI, Digital volume pulse stiffness index; DVP-RI, Digital volume pulse reflection index; GIP, Glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-like peptide-1; GLUT2, Glucose transporter 2; NEFA, Non-esterified fatty acids; TAG, Triacylglycerol; T_{max} , Time of maximum concentration; SGLT1, Sodium-dependent glucose transporter 1.

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[25,26]. A previous RCT showed that a drink containing 600 mg of blackcurrant anthocyanins (1600 mg blackcurrant polyphenols) decreased early postprandial glycemia [27] following the consumption of a starch- and sucrose-rich meal. The present study tested the hypothesis that consumption of drinks containing highly purified polyphenol-rich apple and anthocyanin-rich blackcurrant extracts together with a starch and sucrose meal would reduce the extent of the initial postprandial glycemic response. The primary outcome variable was early postprandial glycemia (incremental area under the curve (iAUC) 0–30 min); secondary outcome variables included other measures of postprandial glycemia (iAUC 0–120 min, C_{max} , T_{max}), parameters of plasma insulin secretion (insulin, C-peptide), incretin response (glucose-dependent insulinotropic polypeptide, GIP), and non-esterified fatty acid (NEFA) responses, and markers of vascular function. A secondary aim was to verify the capacity of the same fruit extracts to inhibit glucose transport *in vitro* using cell culture models, and to investigate the role of SGLT1 and GLUT transporters in any reduction in glucose uptake observed.

2. Materials and methods

2.1. Subjects

Participants were recruited from King's College London and the general public in the London area. Advertisement was done using circular emails within King's College London (KCL), social networking website, poster advertising at KCL, community centers, and *via* an online advertising agency. The intended sample size of 26 subjects had 80% power to detect a difference between average mean area under the curve values (0–30 min) of 0.35 mmol/L·h between test drinks with a significance level of $\alpha = 0.01$ (two-tailed), calculated from a 0.44 mmol/L standard deviation of the difference (using data from our previous study using anthocyanin-rich blackcurrant extract) [27]. A participant information sheet was provided to volunteers who expressed interest; respondents were initially interviewed over the telephone. Inclusion criteria were: healthy men aged 20–60 y and post-menopausal women aged 45–60 y, BMI 18–35 kg/m², able to understand the information sheet and willing to comply with study protocol and able to give informed written consent. Women aged 45 y or older who reported not having had a period for 12 months or longer were defined as postmenopausal. Exclusion criteria were: phenylketonuria, food intolerances, allergies or hypersensitivity, participation in another clinical trial, donation of more than 1500 ml blood in the previous 12 months, full blood counts and liver function test results outside of the normal range, current smokers, or reported giving up smoking within the last 6 months, history of substance abuse or alcoholism, reported history of CVD, diabetes (or fasting glucose ≥ 7.1 mmol/L), cancer, kidney, liver or bowel disease, gastrointestinal disorder or use of drug likely to alter gastrointestinal function, unwilling to restrict consumption of specified high polyphenol foods for 24 h before the study, weight loss >3 kg in preceding 2 months and body mass index <18 or >35 kg/m², blood pressure $\geq 160/100$ mmHg, total cholesterol ≥ 7.5 mmol/L, fasting triacylglycerol concentrations ≥ 5.0 mmol/L, current use of medications that may interfere with the study such as alpha-glucosidase inhibitors (acarbose), insulin-sensitizing drugs (metformin), sulfonyleureas, and lipid-lowering drugs, current use of nutritional supplements that may interfere with the study such as higher dose vitamins/minerals ($>200\%$ reference nutrient intake, B vitamins, vitamin C, calcium, copper, chromium, iodine, iron, magnesium, manganese, phosphorus, potassium and zinc). Eligible participants were invited to a screening visit in the Metabolic Research Unit at the Diabetes & Nutritional Sciences Division, King's College London in a fasting state for measurement of height, weight, waist circumference, % body fat (Tanita Body Composition Analyzer), supine blood pressure and digital volume pulse, liver function tests, hematology, plasma glucose and lipid profile. Subjects who meet all inclusion and exclusion criteria were randomized according to the randomization schedule created using Research Randomizer software (<https://www.randomizer.org>). The allocation of treatment was blinded from the investigators, laboratory technicians and the study participants by an external research technician. The blinding could only be broken in an emergency where it was essential to know which treatment a subject received in order to give the appropriate medical care. Investigators, laboratory technicians and participants remained blinded until after the study was completed and data analysis was performed.

2.2. Study design

A randomized, controlled, double-blind, cross over design was used to allow subjects to receive each of the 3 test drinks in random order at three separate study visits with at least 7 days wash-out between each visit. The study took 7 months to run, from January to July 2015, was sponsored by King's College London and received approval from King's College London ethics committee (REC reference: BDM/14/15–10). This study abided by the principles outlined in the Declaration of Helsinki of 1975 as revised in 1983, and registered at ClinicalTrials.gov (NCT02340039). Drinks were formulated by the authors

and standardized to contain 0 mg polyphenols (CON), 1200 mg apple polyphenols (AE) and 600 mg apple polyphenols+600 mg blackcurrant anthocyanins (AE+BE). Highly purified extracts instead of whole fruits were used in test drinks so to avoid the confounding effect of fruit fiber on gastric emptying rate. The liquid blackcurrant extract (BerryPharma® by Iprona AG, Lana, Italy) contained 1.8% anthocyanins (3.6% total polyphenols). The powdered apple extract (App'lIn by DIANA Food SAS, Antrain, France) contained 67% total polyphenols as analyzed by DIANA Food SAS and confirmed by the Folin–Ciocalteu method in the laboratory at King's College London [28]. Of total apple polyphenols, 40% were flavonoid monomers and phenolic acids (40% flavan-3-ols, 36% dihydrochalcones, 13% flavonols and 11% hydroxycinnamic acids). The extracts were dispersed in a very low-polyphenol double concentrate apple and blackcurrant squash (Robinson's, United Kingdom) made up with water, and drinks were matched for macronutrient and energy content. Each test drink consisted of a fruit drink providing 220 kJ, 0.2 g protein and 12 g carbohydrate, see Table 1. Test drinks (200 ml) were dispensed into identical black bottles and labeled with codes by an external research technician; study investigators and participants were blind to the nature of the contents in each bottle. Drinks were consumed within 2 min immediately followed by consumption of the standardized high-carbohydrate meal consisting of 100 g of white bread (Hovis, London, UK) spread with 30 g of a low-polyphenol apricot jam (Hartley's, Hain Daniels Group, Leeds, UK); both high-carbohydrate foods are low in polyphenols (<6 mg/100 g fresh weight) [29], providing 1284 kJ and 63 g carbohydrate; 41 g starch and 22 g as sugars. Drink and meal were consumed within 5 min; total carbohydrate provided including the drink was 75 g.

On the day previous to each study visit, participants were told not to participate in strenuous exercise and to avoid alcohol, caffeine, oily fish, high-polyphenol and high-nitrate foods (from a list provided), and high-fat foods. Participants were asked to consume a low-fat meal the evening before each visit and arrive following a 12 h fast, avoiding eating or drinking anything except for water until the morning of the study visit. Detailed dietary and lifestyle guidance was provided to each participant before commencing the study period. On arrival to study days, participants were weighed and rested in the supine position for 10 min. Digital volume pulse (DVP) and blood pressure (BP) measurements were recorded for triplicate. For blood sampling a venous cannula was inserted in a vein in the antecubital fossa or a forearm vein by a trained phlebotomist, and baseline samples for analysis of glucose, insulin, C-peptide, triacylglycerol (TAG), NEFA and GIP were collected. Glucose baseline samples were taken in duplicate (at -10 and -5 min). After baseline measurements and blood sampling participants consumed the drink immediately before the high-carbohydrate meal. Blood samples were taken at 10, 20, 30, 45, 60, 75, 90 and 120 min following the first mouthful of the high-carbohydrate meal. BP and DVP measurements were taken again at 60, 90 and 120 min.

Table 1
Energy, nutrient and polyphenol composition of the test drinks

Per 200 ml	AE	AE+BE	CON
<i>Energy and nutrients</i>			
Energy (kJ)	222	227	219
Carbohydrate (g)	12.2	12.2	12.1
Starch (g)	-	-	-
Sugars (g)	12.2	12.2	12.1
of which sucrose (g)	6	6	6
Fat (g)	-	-	-
Protein (g)	0.1	0.2	-
Fiber (g)	0.1	0.4	-
Soluble fiber (g)	0.1	-	-
<i>Polyphenols</i>			
Total phenolics (mg) ¹	1200	1800	<5
Flavonoid monomers (mg)			
Flavan-3-ols (mg) ^{2,3}	293	147	N
Flavonols (mg) ^{2,3}	92	46	N
Quercetin (mg) ^{2,3}	23	12	N
Dihydrochalcones (mg) ^{2,3}	263	131	N
Phlorizin (mg) ^{2,3}	151	76	N
Anthocyanins (mg) ^{3,4}	-	600	<2.5
Phenolic acids (mg)			
Hydroxycinnamic acids (mg) ^{2,3}	79	40	N
Chlorogenic acid (mg) ^{2,3}	47	23	N
Other polyphenols (mg) ¹	473	836	N

AE, 1200 mg of apple polyphenols (1800 mg apple dried extract); AE+BE, 600 mg of blackcurrant anthocyanins (33.3 g blackcurrant liquid extract)+600 mg apple polyphenols (900 mg apple dried extract); CON, 0 mg of BE or AE; Nm negligible amounts.

¹ Estimated from analysis of raw extract by Folin–Ciocalteu assay, not direct analysis of the drinks; ² Analyzed in AE by HPLC-UV; ³ Estimated from Phenol-Explorer and USDA databases for apple and blackcurrant juices from concentrate; ⁴ Analyzed in BE by spectrophotometry.

2.3. Digital volume pulse and blood pressure measurements

Arterial tone was measured using the digital volume pulse (DVP). DVP measurements were obtained by photoplethysmography (PulseTrace PCA 2, Micro Medical Ltd., Kent, UK) and used to calculate stiffness index (DVP-SI, m/s) and reflection index (DVP-RI, %). DVP was measured using an infra-red probe clipped on the finger/thumb of any hand (preferably index finger of the non-dependent hand). Participants were allowed to rest quietly, in supine position, at a comfortable room temperature for 10 min before measurements were performed. The measurements were done 3 times, using the equipment default settings, separated by 30–45 s. The three values for DVP-SI were within 10–15% of each other. If one was clearly irregular it was discarded. Blood pressure was measured at the brachial artery using a calibrated automated blood pressure monitor (Omron 705IT, Omron Healthcare Europe B.V.) immediately after DVP measurements were taken.

2.4. Blood sample analysis

Blood samples were collected into fluoride/oxalate tubes (BD Vacutainer Cat no. 368921) for glucose analysis, into SST serum tubes (BD Vacutainer Cat no. 367954) for insulin, C-peptide, TAG and NEFA analysis and into K₂EDTA tubes for GIP analysis (BD Vacutainer Cat no. 367839). Samples were centrifuged at 1300 g, 4°C for 15 min and plasma/serum aliquots were kept frozen at –40 or –80°C until biochemical analysis. Glucose, TAG and NEFA concentrations were analyzed on the ILab 650 chemistry analyzer using enzymatic assays (IL Test Cat No. 0018259140, IL Triglycerides, Cat No 0018258740 and NEFA Randox Cat. No. FA 115). ELISAs were used to analyze insulin, C-peptide (Siemens Healthcare Diagnostics, Frimley, Surrey, UK) and GIP concentrations (Millipore, MA., USA).

2.5. In vitro studies

Cells were exposed to the same apple extract used in the dietary intervention trial at concentrations in the physiological range estimated to be present in the small intestine after ingestion of test drinks in human studies, in addition to higher and lower concentrations (concentration range 0.07–4.0 mg total polyphenols/ml).

2.5.1. Caco-2 cells

The human intestinal Caco-2 cell line (TC7 subclone) was cultured as previously described [14]. Cells were cultured at 37 °C in a humidified incubator (BIOHIT, HealthCare, UK) in a 5% CO₂–95% air atmosphere in high-D-glucose (25 mM) with glutamine Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), containing 10% fetal bovine serum (Sigma-Aldrich), 50 units penicillin and 50 µg streptomycin (Sigma-Aldrich), 0.1 mM MEM non-essential amino acids (Life Technologies), additional 2 mM L-Glutamine (Life Technologies) and 5 µg/ml Plasmocin (InvivoGen). For glucose transport assays using Caco-2 monolayers, Krebs buffer solution (KBS) containing 30 mM HEPES (Sigma Aldrich), 130 mM NaCl, 4 mM KH₂PO₄, 1 mM MgSO₄·7H₂O, 1 mM CaCl₂, dH₂O, and adjusted pH 7.4, was used as wash media; for sodium-free conditions NaCl was replaced with KCl. Sodium-containing and sodium-free KBS supplemented with 0.2% bovine serum albumin (Sigma Aldrich) was used as pre-incubation and uptake media. Uptake media contained 10 mM D-glucose (BDH Laboratory Supplies) or 10 mM L-glucose (Santa Cruz Biotechnology) and 0.1 µCi/ml Glucose D-[14C(U)] or 0.1 µCi/ml Glucose L-[1-14C] (Perkin Elmer) as tracer. Times for pre-incubation and uptake in all experiments were 15 and 10 min, respectively. Phlorizin, a specific competitive inhibitor of SGLT1 was purchased from Cayman Chemical Company. Phloretin, a specific inhibitor of GLUT2 (which is primarily functional at the apical membrane in the presence of high luminal glucose concentrations) and cytochalasin B, a GLUT1–4 inhibitor were purchased from Santa Cruz Biotechnology. For experiments, cells were seeded at 8000 cell/cm² density in 24-well plates and cultured for 21 d; cells were used between passages 45–53 in all experiments. Assessment of total glucose uptake (the sum of SGLT1- and GLUT-mediated transport) was undertaken in KBS, whereas quantification of GLUT-mediated uptake was undertaken in sodium-free KBS. Before uptake started, DMEM was removed and cells were washed once with room temperature KBS. Cells were pre-incubated for 15 min with pre-incubation media; uptake was initiated by replacing pre-incubation media with uptake media. Uptake media contained, except for controls, glucose transporter inhibitors (phlorizin, phloretin or cytochalasin B) or increasing concentrations of the same apple and blackcurrant extracts used in the clinical trial at physiological concentrations. After 10 min the uptake media was aspirated and the transport process was stopped by washing each well 3 times with ice cold KBS, cells were then processed for radioactivity count on a Liquid Scintillation Counter (Beckman Coulter, LS6500).

2.5.2. Oocytes

Batches of *Xenopus laevis* oocytes were provided by the European Xenopus Resource Centre of the University of Portsmouth. Isolated oocytes were injected with cRNA as previously described [15]. Isolated oocytes of stage V/VI were injected (Nanject II injector, Drummond Scientific Company) with 36.8 nL of rSGLT1 cRNA (1.2–1.5 µg/ml). Oocytes were kept at 19 °C in modified Barth's saline (MBS) media (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 10 mM HEPES, 0.41 mM CaCl₂, 100 U/100 µg penicillin/streptomycin, 0.05 mg/L tetracycline and 0.1 mg/ml

gentamicin, pH 7.5), checked daily and removed into fresh media when necessary. Transport assays were carried out 4 days post-injection, allowing time for the cRNA translation and expression of mature protein (SGLT1 transporter) into the oocyte plasma membrane. Non-injected oocytes were used as shams in the uptake assays and uptake was adjusted for sham diffusional glucose uptake. For glucose transport assays, uptake media contained 10 mM D-glucose and 0.5 µCi/ml Glucose D-[14C(U)] as tracer. MBS media was used as uptake media and phosphate-buffered saline (PBS, Sevens Biotech) as wash media; uptake period in all experiments was 10 min. To ensure the oocyte system was functioning as expected, phlorizin and phloretin were tested to examine the effects of apple and blackcurrant extracts on glucose uptake, uptake media was added containing, except for control, increasing concentrations of apple and blackcurrant extracts at physiological doses. Uptake was initiated by aspirating incubation media and adding 500 µL of uptake medium and terminated by aspirating uptake media and washing three times with 4 ml of ice cold PBS. Subsequently each oocyte was processed for radioactivity counting.

2.6. Statistical analysis

AUC and iAUC were calculated using the trapezoidal rule. iAUC was calculated by subtracting baseline values from all subsequent time-point values [30]. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) v.21. Natural logarithmic transformation was used where data were not normally distributed. Two-way repeated measures analysis of variance (ANOVA) were used for treatment and treatment × time interactions, iAUC 0–30 min, iAUC 0–120 min and C_{max}. A non-parametric Friedman's test was used to detect a significant difference in T_{max} between treatments. P-values were adjusted using two-way repeated measures ANOVA with post hoc analysis by Tukey's adjustment for treatment and treatment × time interactions (GraphPad Prism). In vitro data were analyzed using the software GraphPad Prism 6 for Windows (GraphPad software, CA, USA). Experiments with normally distributed data were analyzed by one-way analysis of variance with Dunnett's multiple comparison post hoc test or by unpaired *t* test. IC50's for cell experiments were estimated by non-linear regression analysis using the software SigmaPlot 13 for windows (Systat Software, CA, USA).

3. Results

A total of 42 healthy men and postmenopausal women aged 20 to 60 years attended screening sessions, 34 met all inclusion criteria and were randomized to treatment. Twenty-five subjects completed the study. Details of study stages from enrolment to completion are shown on the consort diagram (Fig. 1). Baseline characteristics of the 25 participants who completed the study are shown in Table 2.

3.1. Human study

3.1.1. Glucose, insulin, C-peptide and GIP

AE+BE and AE treatments significantly lowered plasma glucose concentrations in the early postprandial period (0–30 min) compared with CON (iAUC 0–30 min, see Table 3 for details). Post hoc analysis with Tukey's adjustment showed significantly lower glucose concentrations following AE and AE+BE compared with CON at 10, 20 and 30 min post-drink (Fig. 2). Changes from baseline for glucose concentrations 0–120 min also differed significantly by treatment; iAUC 0–120 min was significantly different between AE+BE and CON treatments, but not between AE and CON (Table 3). AE and AE+BE had a lowering effect on maximal plasma glucose concentration (C_{max}) when compared with CON: mean difference in C_{max} AE vs. CON (95% CI) –0.7 (–1.3, –0.1), *P*<.05; mean difference AE+BE vs. CON (95% CI) –0.9 (–1.6, –0.3), *P*=.002. T_{max} was greater following AE+BE and AE (medians 60 min, IQR 45, 75, and 45 min, IQR 38,60 respectively) compared with CON (median 30 min, IQR 20, 30, *P*<.0001).

AE+BE and AE treatments significantly lowered insulin and C-peptide concentrations compared with CON (iAUC 0–30 min, see Table 3 for details). Post hoc analysis with Tukey's adjustment for multiple comparisons showed significantly lower (Ln)insulin and C-peptide concentrations following AE and AE+BE compared with CON at 10, 20, 30 and 45 min post-drink, and AE vs. CON for insulin at 120 min (Fig. 3A) and AE+BE vs. CON for C-peptide at 60 min (Fig. 3B). Change from baseline of (Ln)insulin and (Ln)C-peptide concentrations 0–120 min also differed significantly by treatment; iAUC 0–120 min was significantly different between treatments when comparing

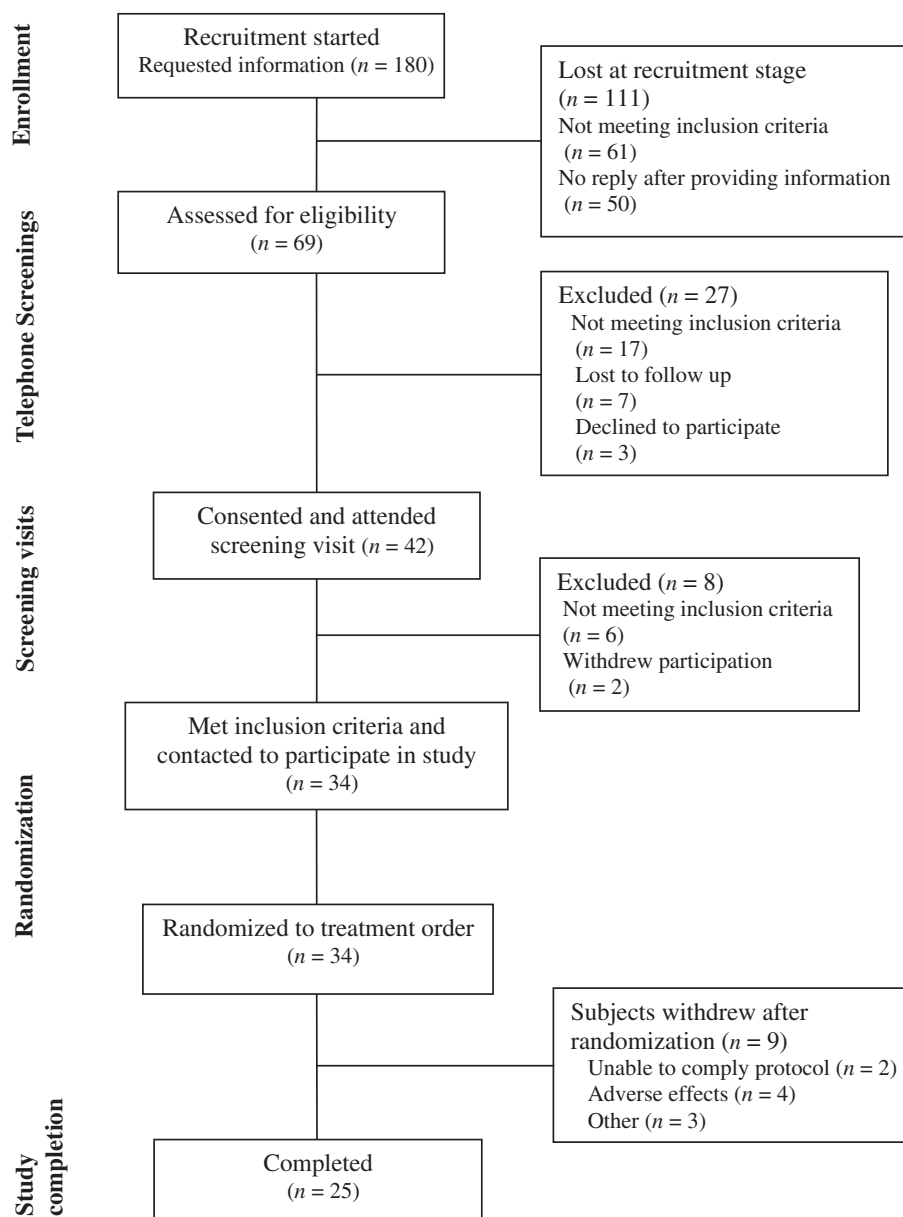


Fig. 1. CONSORT flow diagram of the study participants.

AE+BE and AE with CON for insulin and AE+BE only compared with CON for C-peptide (Table 3). AE+BE had a lowering effect on maximal plasma (*Ln*)insulin concentration (C_{\max}) when compared with CON: C_{\max} mean difference AE+BE vs. CON (95% CI) -16.8 ($-33.5, -0.11$), $P < .05$. There were no differences in C_{\max} for C-peptide between drinks. T_{\max} was significantly longer following polyphenol-rich drinks compared with CON for both insulin and C-peptide. Insulin T_{\max} was greater following AE+BE and AE (medians 75 min, IQR 60, 75, and 60 min, IQR 45, 75 respectively) compared with CON (median 45 min, IQR 25, 60, $P < .0001$). C-peptide T_{\max} was greater following AE+BE and AE (medians 75 min, IQR 60, 90, and 75 min, IQR 60, 105, respectively) compared with CON (median 60 min, IQR 45, 75, $P < .0001$).

AE+BE and AE treatments significantly lowered GIP concentrations compared with CON (iAUC 0–30 min, see Table 3 for details). Post hoc analysis with Tukey's adjustment for multiple comparisons showed significantly lower GIP concentrations following AE and AE+BE compared with CON at all time points up to 75 min (Fig. 4). Change from baseline of (*Ln*)GIP concentrations 0–120 min also

differed significantly by treatment; iAUC 0–120 min was significantly different between AE+BE, AE and CON. There was a significant decrease in C_{\max} following AE+BE and AE compared with CON (mean differences (95% CI) -45.5 ng/L ($-73.6, -17.5$), $P < .005$, and -32.7 ng/L ($-54.1, -11.4$), $P < .01$, respectively). T_{\max} was greater following AE+BE and AE (medians 90 min, IQR 83, 120, and 90 min, IQR 75, 120, respectively) compared with CON (median 60 min, IQR 45, 75, $P < .0001$).

3.1.2. NEFA and TAG

AE+BE inhibited the decreases in plasma NEFA concentrations throughout the 120 min of postprandial period; iAUC 0–120 min was significantly different between AE+BE and CON, mean difference (95% CI) 17.2 mmol/L min (7.2, 27.3), $P < .005$. Post hoc analysis with Tukey's adjustment for multiple comparisons showed significantly higher NEFA concentrations following AE and AE+BE compared with CON from 20 min up to 120 min (Fig. 5). There was a significant increase in C_{\max} following AE+BE vs. CON (mean difference (95% CI) -0.14 mmol/L ($-0.23, -0.05$), $P < .005$). There were no significant

Table 2
Baseline characteristics of the study population (n=25)

Variable ¹	
Age (y)	32.3 (14.4)
Males	26.1 (7.5)
Females	57.2 (3.4)
Sex (male to female ratio)	20:5
Body Mass Index (kg/m ²)	23.5 (2.8)
Systolic blood pressure (mmHg)	112.2 (10.8)
Diastolic blood pressure (mmHg)	69.2 (7.9)
Waist circumference (cm)	
Males	81.9 (7.4)
Females	85.8 (11.4)
Body fat (%)	
Males	16.4 (5.8)
Females	34.9 (5.5)
Fasting plasma glucose (mmol/L)	5.1 (0.3)
Fasting plasma triacylglycerol (mmol/L)	1.1 (0.9)
Fasting plasma total cholesterol (mmol/L)	4.3 (1.0)
Fasting plasma LDL cholesterol (mmol/L)	2.6 (0.9)
Fasting plasma HDL cholesterol (mmol/L)	
Males	1.3 (0.3)
Females	1.6 (0.4)

¹ Values are means (SD).

differences in T_{max} . There were no differences between treatments for plasma TAG concentrations (Supplementary Fig. 1).

3.1.3. Blood pressure and vascular function

There were no differences between treatments for systolic and diastolic blood pressure (Supplementary Fig. 2). There were no differences in raw data for stiffness and reflection index values, however change from baseline on DVP-RI values were significantly reduced during the whole postprandial period by treatment AE compared with CON (Supplementary Fig. 3).

3.2. In vitro studies

3.2.1. Caco-2 cells

Dose–response assays in the presence of different concentrations of apple polyphenols showed an inhibition on total and GLUT-mediated glucose uptake down to a concentration of 0.15 mg polyphenols/ml ($P<0.0001$) (Fig. 6A and B). A concentration of 0.3 mg polyphenols/ml inhibited 51% of total glucose uptake and 46% of sodium-independent uptake, and corresponded to an estimated effective physiological dose in the human study of 600 mg apple polyphenols (900 mg apple extract). Similar dose–response inhibitory

effects of blackcurrant extract on total and sodium-independent glucose uptake were observed, down to a concentration of 0.2 mg polyphenols/ml ($P<0.0001$) (Fig. 7A and B). Concentrations of blackcurrant polyphenols necessary to inhibit total and sodium-independent glucose uptake by half relative to control (0 mg polyphenols) were estimated (IC₅₀). Blackcurrant polyphenols under sodium-dependent conditions (total glucose uptake) had an IC₅₀ of 0.51 ± 0.07 mg polyphenols/ml (mean \pm SE) and under sodium-independent conditions an IC₅₀ of 0.63 ± 0.07 mg polyphenols/ml, suggesting a slightly greater inhibition of total glucose uptake compared with GLUT-mediated glucose uptake.

3.2.2. Oocytes

In oocytes injected to express SGLT1, glucose uptake was inhibited by 85% and 59% by phlorizin and phloretin (0.5 mM), respectively, relative to control treatment ($P<0.0001$) (data not shown). There was a highly significant relative reduction in glucose uptake after 0.125–4.0 mg apple polyphenols/ml (Fig. 6C) and 0.1–1.6 mg blackcurrant polyphenols/ml ($P<0.0001$) (Fig. 7C), with no dose–response effect for apple extract and an IC₅₀ of 0.12 mg blackcurrant polyphenols/ml following blackcurrant extract treatment.

4. Discussion

Consumption of drinks containing apple polyphenols and the combination of apple with blackcurrant polyphenols exerted an inhibitory effect on postprandial glycemia and insulinemia, particularly during the first 30 min post-meal, with the combination of both extracts having a more potent effect. A similar response was found for the gut incretin, GIP, following both treatments. Previously we reported that a drink containing 600 mg of blackcurrant anthocyanins inhibited plasma glucose and insulin early response 0–30 min. By combining the same dose with 600 mg of apple polyphenols in a test drink, this inhibitory effect can be increased further. The apple extract used in the current study was rich in phlorizin (8.4% of the extract), giving a total of 151 and 76 mg in the AE and AE+BE drinks, respectively. In previous reports, a cloudy apple juice containing 26 mg of phlorizin, 25 g glucose and 30.7 g fructose significantly lowered plasma glucose in human volunteers compared with a control drink, as demonstrated by a lower glucose iAUC 0–30 and 30–90 min [23]. Furthermore, a dose of 2.8 g of an apple extract containing 448 mg of phlorizin, reduced plasma glucose iAUC 0–45 min in healthy human subjects following a 75 g glucose load [8], and 25 g of unripe apple extract, derived from apple pomace, containing 315 mg of phlorizin reduced postprandial glucose response in healthy volunteers following a 50 g glucose load [24]. The magnitude of the inhibition found in

Table 3
Effects of apple, apple and blackcurrant and placebo test drinks in incremental area under the curve (iAUC) 0–30 and 10–120 min for plasma glucose, insulin, C-peptide and GIP in the study population (n=25)

	Treatment comparison	iAUC (0–30 min)			iAUC (0–120 min)		
		Mean difference	95% CI ¹	P value ¹	Mean difference	95% CI ¹	P value ¹
Glucose (mmol/L·min)	AE–CON	–26.3	–34.9, –17.7	<0.0005	–29.8	–78.1, 18.4	0.363
	AE+BE–CON	–31.5	–40.8, –22.2	<0.0005	–51.6	–93.8, –9.4	0.014
	AE+BE–AE	–5.2	–9.8, –0.6	0.023	–21.7	–65.9, 22.5	0.638
Insulin (mU/L·min)	AE–CON	–613.1	–771.6, –454.6	0.000	–872.7	–1734.4, –11.1	0.047
	AE+BE–CON	–675.3	–915.1, –435.5	0.000	–1109.7	–1642.2, –577.2	0.000
	AE+BE–AE	–62.2	–203.0, 78.6	0.782	–236.9	–1083.1, 609.2	0.565
C-peptide (pmol/L·min)	AE–CON	–9221.9	–11,031.5, –7142.3	0.000	–14,219.7	–31,227.2, 2787.8	0.122
	AE+BE–CON	–11,881.4	–15,099.3, –8663.6	0.000	–27,941.8	–44,418.5, 11,465.1	0.001
	AE+BE–AE	–2659.5	–4817.5, –501.6	0.013	–13,722.1	–31,931.5, 4487.3	0.188
GIP (ng/L·min)	AE–CON	–1272.7	–1568.3, –977.2	0.000	–4038.1	–5467.5, –2608.8	0.000
	AE+BE–CON	–1735.3	–1998.1, –1472.6	0.000	–6097.2	–7742.5, –4451.9	0.000
	AE+BE–AE	–462.6	–617.2, –307.9	0.000	–2059.0	–3151.2, –966.9	0.001

AE, 1200 mg of apple polyphenols; AE+BE, 600 mg of apple polyphenols+600 mg blackcurrant anthocyanins; CON, 0 mg of blackcurrant anthocyanins and apple polyphenols.

¹ From two-way analysis of variance, confidence interval and P-value adjusted by Bonferroni's procedure for multiple comparisons, n=25.

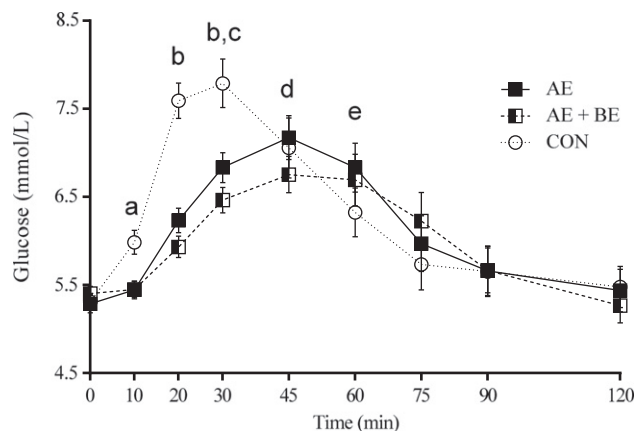


Fig. 2. Postprandial glucose concentrations. Mean (\pm SEM) plasma glucose concentrations following ingestion of two test (AE, AE+BE) and the control (CON) drinks before consuming a mixed carbohydrate meal, in randomized order ($n=25$). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols+600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline ($P<.005$). Post hoc analysis of time-point differences in change from baseline in glucose with Tukey's adjustment: ^a $P<.05$ CON compared with AE and AE+BE; ^b $P<.0001$ CON compared with AE and AE+BE; ^c $P<.0001$ AE compared with AE+BE and CON; ^d $P<.05$ AE compared with AE+BE; ^e $P<.05$ CON compared with AE.

the present study is greater than that previously reported in the literature. Possible explanations include the solid state of the high-carbohydrate meal prolonging digestion (thereby allowing more time for inhibition to take effect) compared with liquid meals, and the use of a mixed carbohydrate meal (starch and sucrose) as opposed to single carbohydrate loads of glucose, fructose, and sucrose in studies reported previously [8,24].

In vitro studies have shown inhibitory effects on the digestive enzymes, α -amylase and α -glucosidases (maltase and sucrase), by flesh and peel apple extracts [31] and also some of the principal individual polyphenols present in apple, such as quercetin [10], proanthocyanidins [32] and chlorogenic acid [33]. Furthermore, inhibition of glucose transporters (SGLT1 and GLUT2) by apple extracts, phlorizin, and quercetin glycosides [8,16,34], may also contribute to the marked effect on postprandial glucose and insulin response observed *in vivo*. Our *in vitro* data supports the theory that the apple extract in the present human study may have exerted an inhibitory effect on small intestinal glucose transport, since the same extract dose-dependently inhibited total glucose uptake and sodium-independent glucose uptake into Caco-2 cells, a well-established *in vitro* model of the human enterocyte. Inhibition of glucose uptake in Caco-2 cells by apple extract appeared to occur predominantly *via* inhibition of the sodium-independent route, most likely mediated by GLUTs. However, components of the apple extract may have also inhibited the sodium-dependent route, since the extract significantly inhibited glucose uptake into oocytes injected to express SGLT1. Although phlorizin is an effective inhibitor of SGLT1-mediated glucose transport, other polyphenols contained in apple extract also have inhibitory effects [8] and it is likely that larger inhibitory effects on glucose uptake may be exerted by complex mixtures of polyphenols in fruit extracts due to additive or synergistic effects. Proanthocyanidins, estimated to constitute 540 and 600 mg of the AE+BE and AE drinks, respectively [29], are probably responsible for a significant proportion of the inhibitory effects on postprandial glucose response [35]. *In vitro* enzymatic studies have suggested non-specific inhibition of α -amylase by proanthocyanidins [7]. Interactions between polyphenols might show an additive effect when proanthocyanidins are combined with other phenolic compounds which inhibit α -amylase, such as anthocyanins [36], flavonols and flavones [37], or a synergistic

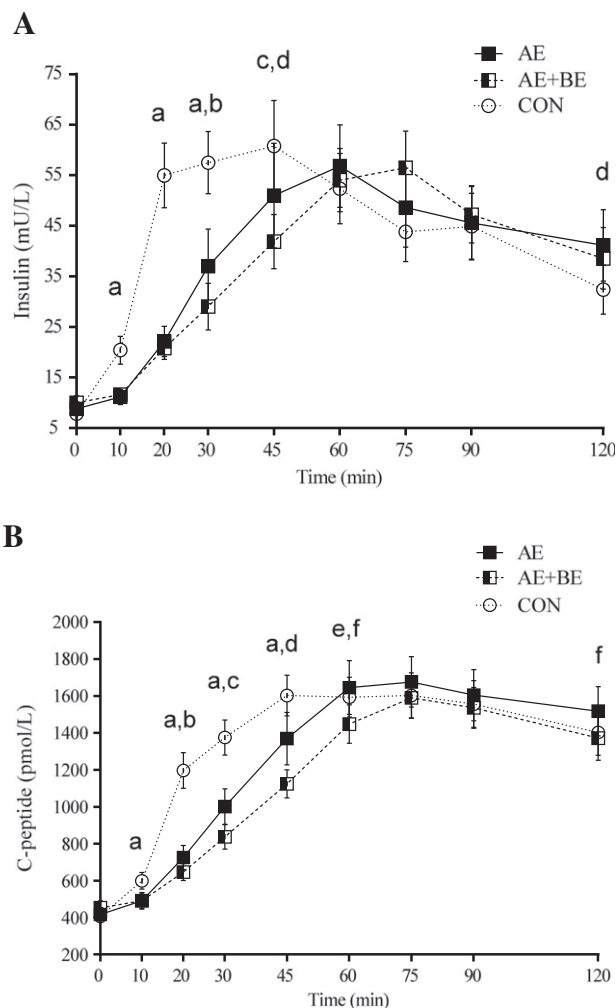


Fig. 3. Postprandial insulin and C-peptide concentrations. Mean (\pm SEM) plasma insulin (A) and C-peptide (B) concentrations following ingestion of two test (AE, AE+BE) and the control (CON) drinks before consuming a mixed carbohydrate meal, in randomized order ($n=25$). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols+600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. (A) There was an overall treatment effect on changes from baseline ($P<.001$). Post hoc analysis of time-point differences in change from baseline in insulin compared with Tukey's adjustment: ^a $P<.0001$ CON compared with AE and AE+BE; ^b $P<.0005$ AE compared with AE+BE; ^c $P<.0001$ CON compared with AE+BE; ^d $P<.05$ CON compared with AE. (B) There was an overall treatment effect on changes from baseline ($P<.0001$). Post hoc analysis of time-point differences in change from baseline in C-peptide with Tukey's adjustment: ^a $P<.0001$ CON compared with AE and AE+BE; ^b $P<.05$ AE compared with AE+BE; ^c $P<.0001$ AE compared with AE+BE; ^d $P<.0005$ AE compared with AE+BE; ^e $P<.0005$ CON compared with AE+BE; ^f $P<.05$ AE compared with AE+BE.

effect when combined with α -glucosidase inhibitors such as chlorogenic acid [38].

Plasma insulin concentrations follow the glycemic response curve, but circulating concentrations are subject to the rate of hepatic extraction as well as pancreatic secretion rates. We analyzed C-peptide concentrations (a polypeptide removed from the proinsulin molecule to produce insulin), as a more accurate biomarker of insulin secretion rates [39]. Results confirm a potent inhibition of insulin secretion up to 45 min post-meal by AE+BE, and to a lesser extent AE. Plasma NEFA concentrations followed the expected insulin-mediated suppression following a high-carbohydrate meal following each of the test drinks, but the degree of suppression was ameliorated following AE and AE+BE treatments compared with CON, mirroring the reduction in plasma insulin and GIP secretion.

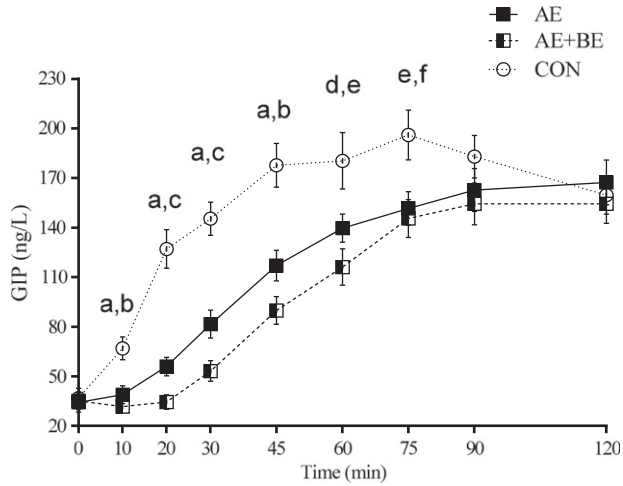


Fig. 4. Postprandial GIP concentrations. Mean (\pm SEM) plasma GIP concentrations following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomized order ($n=25$). AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols+600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline ($P<.0001$). Post hoc analysis of time-point differences in change from baseline with Tukey's adjustment: ^a $P<.00001$ CON compared with AE and AE+BE; ^b $P<.05$ AE compared with AE+BE; ^c $P<.00001$ AE compared with AE+BE; ^d $P<.00001$ CON compared with AE+BE; ^e $P<.05$ CON compared with AE; ^f $P<.05$ CON compared with AE+BE.

Inhibition of incretin secretion is a prime mechanistic candidate for the inhibition in early-phase insulin secretion. Plasma concentrations of the incretin, GIP, were decreased by treatments AE+BE and to a lesser extent AE up to 75 min postprandially, in agreement with previous findings following a drink containing blackcurrant extract [27]. Intestinal sodium-dependent glucose transporter SGLT1 has been shown to be physically and physiologically related with the expression of GIP, and SGLT1 may act as a glucose sensor for GLP-1 secretion [40]. GIP is secreted in the proximal section of the small intestine by K-cells only minutes after carbohydrate and fat intake [41]. Our results agree

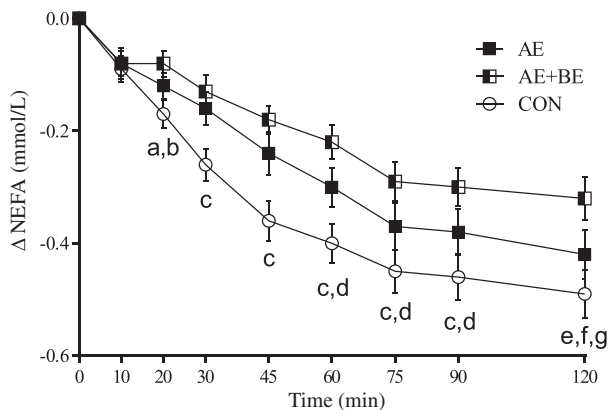


Fig. 5. Postprandial non-esterified fatty acid concentrations. Mean (\pm SEM) plasma NEFA concentrations following ingestion of 3 low sugar fruit drinks immediately before consuming a mixed carbohydrate meal, in randomized order ($n=25$). Baseline values (mmol/L): AE 0.47 ± 0.05 , AE+BE 0.40 ± 0.04 , CON 0.54 ± 0.04 . AE; 1200 mg of apple polyphenols, AE+BE; 600 mg of apple polyphenols+600 mg blackcurrant anthocyanins, CON; 0 mg of blackcurrant anthocyanins and apple polyphenols. There was an overall treatment effect on changes from baseline ($P<.001$). Post hoc analysis of time-point differences in change from baseline in NEFA with Tukey's adjustment: ^a $P<.00005$ CON compared with AE+BE; ^b $P<.05$ CON compared with AE; ^c $P<.00001$ CON compared with AE and AE+BE; ^d $P<.05$ AE compared with AE+BE; ^e $P<.00001$ CON compared with AE+BE; ^f $P<.00005$ CON compared with AE; ^g $P<.0005$ AE compared with AE+BE.

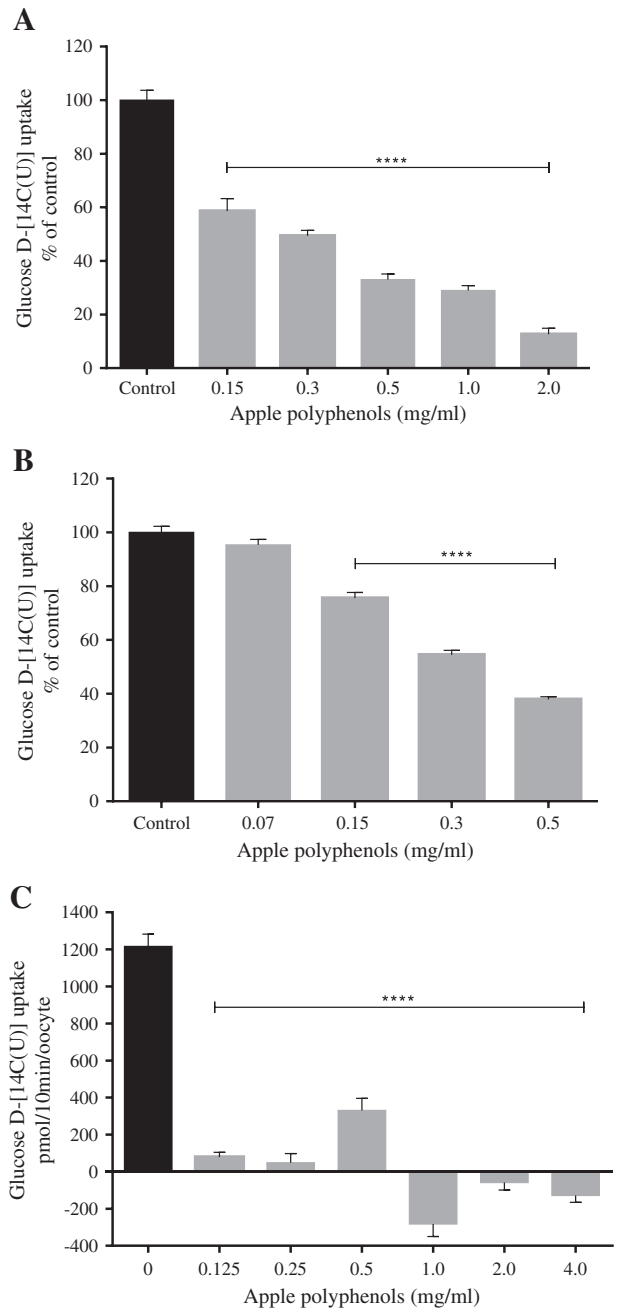


Fig. 6. Acute effects of apple extract on total and GLUT-mediated glucose transport in Caco-2 cells (A and B), and SGLT-mediated glucose uptake in *Xenopus* oocytes (C). Caco-2/TC7 cells and *Xenopus* oocytes injected to express SGLT1 were treated with increasing concentrations of apple polyphenols contained in uptake media. Values were corrected for simple diffusion by subtracting L-glucose uptake in the case of Caco-2 cells, and corrected for diffusional glucose uptake in oocytes by subtracting sham oocytes uptake. Data are presented as mean \pm S.E.M. ($n=3-5$). One-way analysis of variance followed by multiple comparisons against control with Dunnett's adjustment: (A) Total glucose uptake in Caco-2/TC7 cells: $****P<.0001$ for the difference between control and 0.15, 0.3, 0.5, 1.0 and 2.0 mg apple polyphenols/ml treatments. (B) GLUT-mediated glucose uptake in Caco-2/TC7 cells: $***P<.0005$ for the difference between control and 0.15, 0.3 and 0.5 mg apple polyphenols/ml treatments. (C) SGLT-mediated uptake in oocytes: $****P<.0001$ for the difference between 0 mg/ml and 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg apple polyphenols/ml.

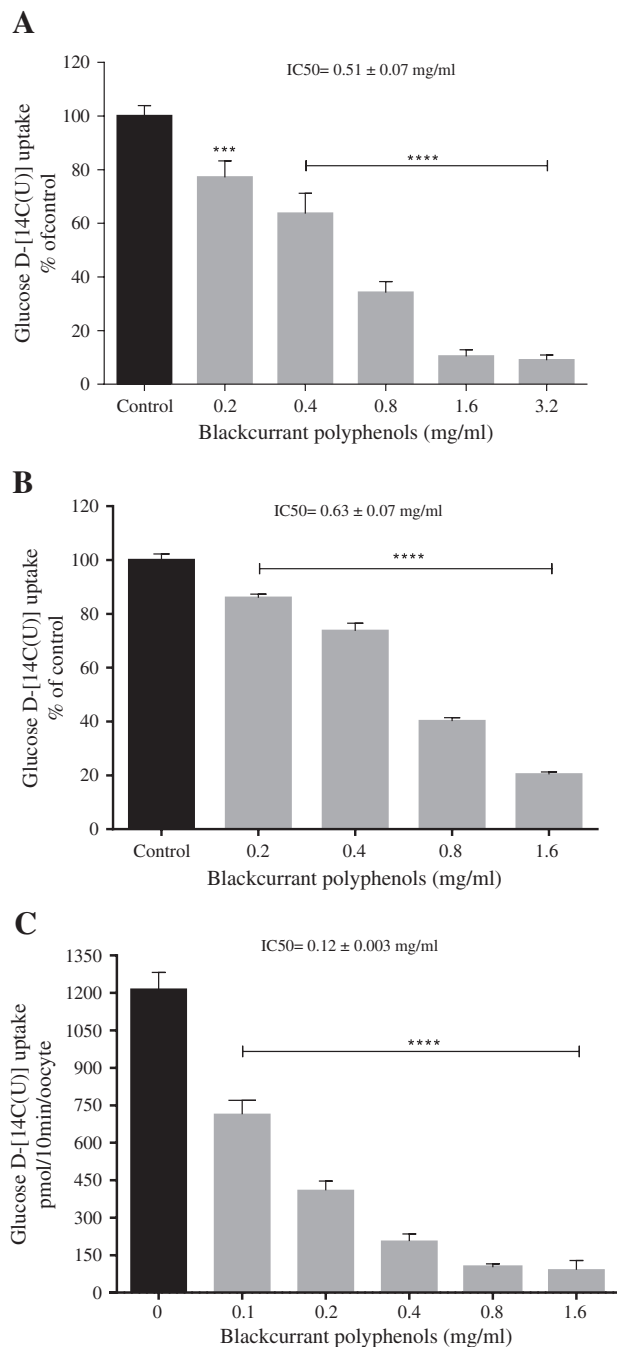


Fig. 7. Acute dose response effects of blackcurrant extract on total and GLUT-mediated glucose transport in Caco-2 cells (A and B), and SGLT-mediated glucose uptake in *Xenopus* oocytes (C). Caco-2/TC7 cells and *Xenopus* oocytes injected to express SGLT1 were treated with increasing concentrations of blackcurrant polyphenols contained in uptake media. Values were corrected for simple diffusion by subtracting L-glucose uptake in the case of Caco-2 cells, and corrected for diffusional glucose uptake in oocytes by subtracting sham oocytes uptake. Data are presented as mean ± S.E.M. ($n=3-5$). One-way analysis of variance followed by multiple comparisons against control with Dunnett's adjustment: (A) Total glucose uptake in Caco-2/TC7 cells: *** $P<.001$ for the difference between control and 0.2 mg blackcurrant polyphenols/ml; **** $P<.0001$ for the difference between control and 0.4, 0.8, 1.6 and 3.2 mg blackcurrant polyphenols/ml treatments. (B) GLUT-mediated uptake conditions in Caco-2 cells: **** $P<.0001$ for the difference between control and 0.2, 0.4, 0.8 and 1.6 mg blackcurrant polyphenols/ml treatments. (C) SGLT-mediated uptake in oocytes: **** $P<.0001$ for the difference between 0 mg/ml and 0.1, 0.2, 0.4, 0.8, and 1.6 mg blackcurrant polyphenols/ml. Nonlinear regression analysis was used to estimate IC50 values.

with a previous report of inhibition of glucose-induced, SGLT1-mediated incretin secretion by phlorizin in mice [42], although experiments using a rat model suggest GLUT2 might also play a role as glucose sensor for glucose-induced incretin secretion [43]. This presents an additional mechanism whereby polyphenols such as phloretin, a GLUT2 inhibitor, may influence gut hormone secretion. Although phloretin was only present in low amounts in this apple extract, phlorizin could be rapidly cleaved by the enzyme lactase phlorizin hydrolase (LPH) to release the aglycone phloretin.

High dietary glycemic index and glycemic load independently increase risk of T2D [44]. Frequent elevated excursions in postprandial glucose concentrations are thought to increase risk of T2D and CVD by inducing oxidative stress and glycation of proteins [5]. Studies with acarbose (an inhibitor of α -glucosidase) show that reducing the rate of carbohydrate digestion can be as effective as metformin (an insulin-sensitizing drug) in reducing glycosylated hemoglobin [45], and that acarbose therapy can also reduce the risk of progression to diabetes in participants with impaired glucose tolerance by 25% [46]. The preventative effects of pharmacological therapies that inhibit the rate of carbohydrate digestion and absorption suggests that mechanistically analogous dietary strategies to control postprandial hyperglycemia could strengthen efforts to reduce the risk of T2D.

Strengths of this study include the robust study design and the use of highly purified fruit extracts removing the confounding factor of fruit fiber. The dose of blackcurrant extract used is achievable in everyday diets, as intake of 600 mg of blackcurrant anthocyanins is equivalent to ~100 g of fresh blackcurrants [29]. In treatment AE+BE, the blackcurrant extract was combined with apple extract containing 600 mg apple phenolics. An intake of 600 mg apple phenolics is equivalent to ~300 g of fresh whole dessert apples (1.5–2 average (182 g) medium-sized apples [29,47]), and the AE treatment was equivalent to twice this amount, which is physically possible to consume but unusual in single sittings. However, in this case, the apple extract was particularly concentrated in phlorizin; for example, the AE treatment provided an amount equivalent to approximately 31 medium-sized unpeeled dessert apples [29]. Phlorizin is present in more concentrated amounts in apple stem (pedicel), seeds and skin (exocarp), which are often disposed of before eating. AE+BE contained a higher amount of total phenolic content than AE, but half as much phlorizin. Due to the diverse complexity in function-structural properties of polyphenols it is arguable that the different doses of total phenolics are not a hindrance to interpretation of the results. The combination of fruit extracts, and the potential presence of un-identified polyphenols contained within them, makes it difficult to attribute effects on glucose homeostasis entirely or even partially to specific phenolic acids or flavonoids. However, combinations of polyphenol-rich fruit extracts may exert potent effects on glucose absorption that may be additive or even synergistic in nature.

5. Conclusions

In conclusion, ingestion of a large dose of apple polyphenol-rich extract, and half of this dose in combination with blackcurrant anthocyanin-rich extract, decreased postprandial glycemia following a starch- and sucrose-containing meal and inhibited secretion of insulin and an incretin (GIP). A mixture of apple and blackcurrant polyphenols had a more potent effect than apple polyphenols alone. Equivalent intakes of apple polyphenols, to the doses used in our study are challenging to achieve by consumption of fresh fruit, nevertheless, since apples are consumed frequently in the general population and are extensively available, the cumulative impact of daily small inhibitory effects on postprandial glycemia could potentially have an impact on long-term health. Although availability of blackcurrants is more limited, other berries more widely consumed, like strawberries, grapes, raspberries, blackberries, blueberries, and cranberries, contain

varying profiles of anthocyanins and proanthocyanidins that are likely to exert similar effects. These results provide evidence for one of many routes by which cardio-metabolic protection may occur if dietary guidelines for fruit and vegetable intakes are adhered to, particularly if combinations are consumed shortly before, or possibly in combination with, high-carbohydrate meals.

Author contributions

M. L. C. A. conceived and designed the study, conducted the clinical trial and in vitro research, collected and analyzed data, performed statistical analysis and wrote the manuscript; S. G. S., J. E. M., R. K. M., C-I. F. and G. N. L-G performed the clinical trial research, collected and analyzed data; W. L. H. conceived and designed the study, participated in the interpretation of data and revision of the manuscript; C. P. C contributed to the methodology and design of the cell experiments. All authors read and approved the final draft.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2017.07.013>.

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